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TECHNICAL MANUSCRIPT 469

AN AUTOMATED HEMAGGLUTINATION TECHNIQUE FOR ESTIMATING THE ACTIVITY OF TYPE A BOTULINUM TOXIN

James B. Williams

JUNE 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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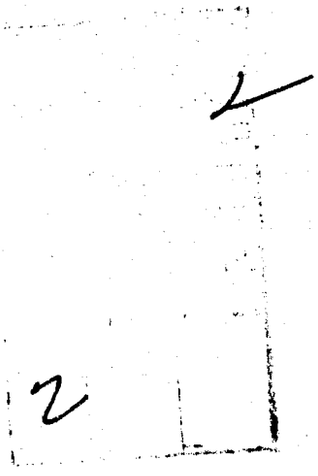

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AN AUTOMATED HEMAGGLUTINATION TECHNIQUE FOR ESTIMATING THE ACTIVITY OF TYPE A BOTULINUM TOXIN

James B. Williams

Process Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1B522301A082

June 1968

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ACKNOWLEDGMENTS

I acknowledge the helpful assistance of Robert L. Willard and Ivan Lantz of Fort Detrick and Roebert Stearman of C-E-I-R, Inc.

I also thank Francis J. Weirether and Edward J. Schantz of Fort Detrick for supplying Clostridium botulinum cultures and crystalline toxin, respectively, and Murray S. Cooper, Lederle Laboratories, for supplying specific antitoxin.

ABSTRACT

A quantitative relationship between hemagglutination titer and animal toxicity of type A botulinum toxin was demonstrated utilizing a modification of the hemagglutination procedure of Rosenfield et al. Hemagglutination titer was correlated to animal toxicity by measuring hemagglutinin activity of known concentrations of toxic material with an established mouse intraperitoneal LD_{50} value. The automated procedure employed is accurate and reproducible in the range of 5 to 50 μg toxin per ml.

CONTENTS

FIGURES

TABLES

I. INTRODUCTION

Estimation of the potency of botulinal toxin usually depends upon a dose-response relationship in animals. Tests such as the Oudin are not necessarily reliable indexes of toxic potency because toxicity decays more easily than antigenicity. Another objection to immunodiffusion tests is the presence of false precipitin bands that may result from impurities in the toxin. Lamanna and Lowenthal[1] showed that crystalline type A toxin produces two precipitin bands with specific antisera, but only one when the hemagglutinin has been absorbed from the toxin. They also observed that a cross reaction occurs between type A hemagglutinin and specific B antitoxin.

Because of the limitations of in vitro tests for botulinal toxin, most investigations to improve assay procedures have been directed toward improvement of the animal test. Such tests are frequently affected by inconsistent results. The development of laboratory identification procedures not dependent upon animal tests would save much time and eliminate costly assay procedures.[2]

The agglutinating properties of type A toxin were investigated by Lamanna[3] and Lowenthal and Lamanna[4,5] as a possible indicator of toxic activity. They observed that under the influence of varying physical factors, the relationship between toxicity and hemagglutination is not constant and agglutinating capacity does not necessarily indicate activity of toxin in all cases. However, if these physical factors are controlled within a standard set of conditions, one may hypothesize that for a given hemagglutination titer, a precise amount of toxicity will be represented. By using a toxic material with an established mouse intraperitoneal (MIP) LD_{50} value, it seems possible to correlate hemagglutination titers with LD_{50} values. Such data might then be used to prepare a standard hemagglutination curve in which the LD_{50} value could be interpreted at any point.

Rosenfield, Szymanski, and Kochwa[6] designed a system of automatic analysis in which the parameters affecting hemagglutination could be closely controlled. This system, with some modification in design and reagent, appeared applicable to a more detailed study of botulinal hemagglutination. The objective of this investigation was to determine whether, in this system, a standard procedure could be developed to establish correlations between hemagglutination titer and $MIPLD_{50}$ values of type A botulinum toxin.

II. MATERIALS AND METHODS

A. TOXIN

Cultures were grown either in thioglycollate or N-Z-Amine, type B medium from a trypticase agar stab subculture of Clostridium botulinum, strain A, FD-SCC-D419, obtained from Mr. Francis J. Weirether, Process Development Division, Fort Detrick. Partially purified toxin in dry form was prepared by acid precipitation of an N-Z-Amine, type B culture with subsequent drying. Purified crystalline type A toxin was obtained from Dr. Edward J. Schantz, Physical Science Division, Fort Detrick.

B. ANTITOXIN

Specific type A equine antitoxin (300 guinea pig units/ml) was obtained from Dr. Murray S. Cooper, Lederle Laboratories, Pearl River, New York.

C. AUTOMATED EQUIPMENT

Technicon Autoanalyzer equipment was obtained from Technicon Instruments Corp., Ardsley, New York. A flow diagram of the system is shown in Figure 1.

D. REAGENTS

Phosphate buffered saline (PBS), 0.15 M, pH 6.8, was prepared by adding 375 ml 0.15 M KH_2PO_4 and 625 ml of 0.15 M Na_2HPO_4 to 1 liter of physiological saline.

Normal rabbit blood was defibrinated by stirring in a beaker with a footed plastic rod. The erythrocytes were then washed with PBS, centrifuged, and decanted. This procedure was repeated three times. The remaining cells were resuspended to a 20% v/v solution of 0.5% Ficoll (Technicon) in PBS.

A 1.5% w/v solution of polyvinylpyrrolidone (PVP K-90 Technicon) was prepared in PBS.

A 0.5% v/v solution of Triton X-100 (Rohm & Haas) was prepared in distilled water.

E. HEMAGGLUTINATION

The following conditions were established for the automated hemagglutination procedure based upon the report of Rosenfield et al.[8] and other unpublished data from our laboratory.

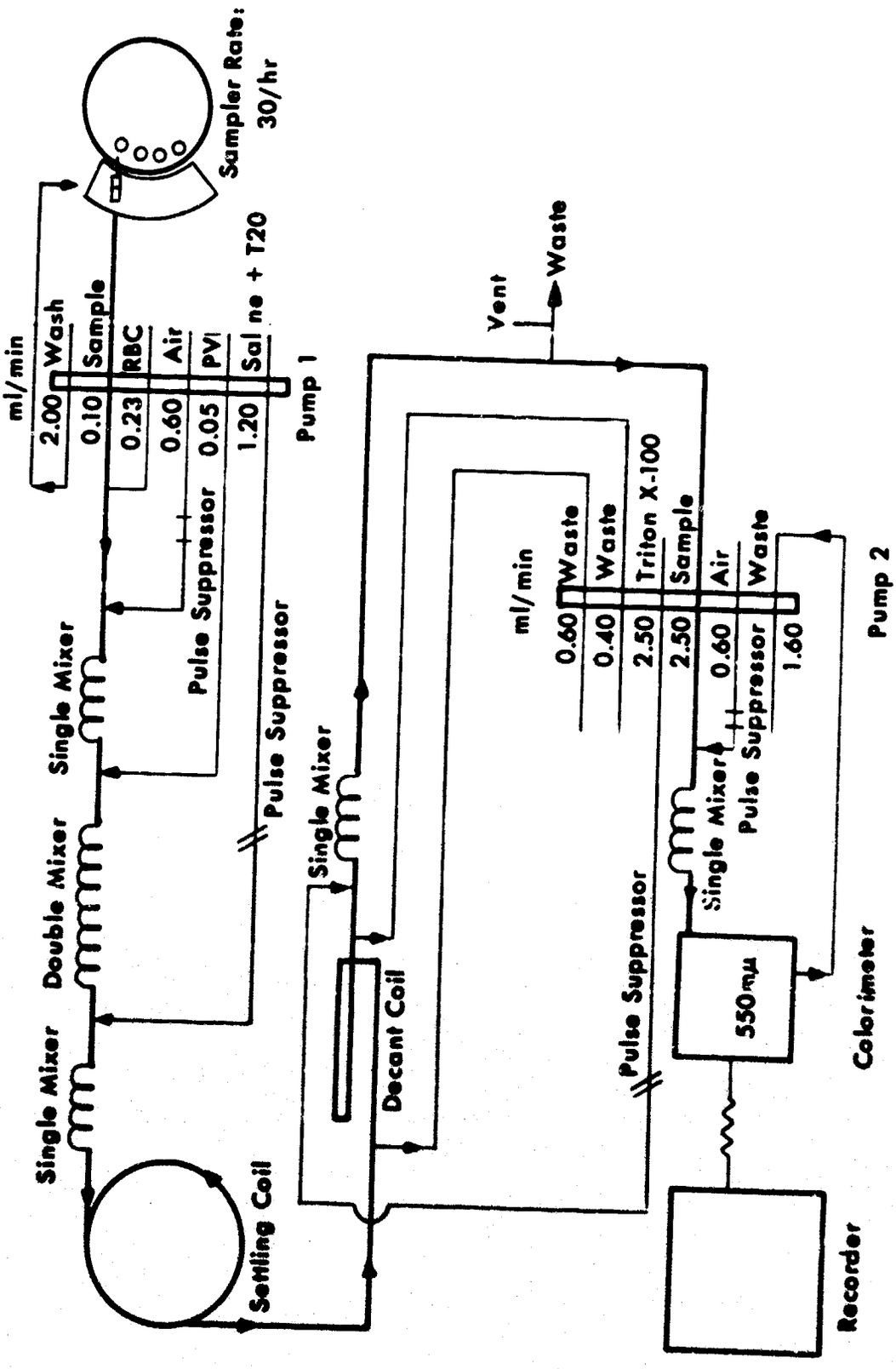

Figure 1. Flow Diagram of the Hemagglutination System.[6]

The assays were performed at ambient temperature. Samples were mixed with the erythrocyte suspension for approximately 3 minutes to permit adsorption of toxin. Then polyvinylpyrrolidone was added to induce rouleau formation. After further mixing of sample and reagent for 5 to 6 minutes, PBS containing 1 drop Tween 20 per liter (Atlas Powder Co.) was introduced into the system to resuspend nonagglutinated cells. The agglutinated cells were allowed to fall out in a settling coil and were removed by siphoning. The nonagglutinated cells were lysed by introducing the Triton X-100 solution. The resulting hemoglobin color was read at 550 mμ and recorded. Quantitation was based on the hemoglobin content of nonagglutinated erythrocytes.

F. HEMAGGLUTINATION INHIBITION

Samples of toxin were prepared in PBS from partially purified dried material. Antitoxin was serially diluted with PBS to 1:512. Equal volumes of these dilutions were added to equal volumes of the toxin solution and allowed to react for 45 minutes at ambient temperature. The samples were then assayed as in the hemagglutination technique.

G. ANIMAL TESTS

Homogeneous Swiss white mice (Animal Farm, Fort Detrick), 16 to 20 g, were inoculated intraperitoneally with 0.5-ml samples of toxin. For LD_{50} determinations, five dilution points were used for each sample, 10 mice for each point. Dilutions of samples were prepared using 0.2% gel-phosphate diluent. All animals were held for at least 4 days. A probit analysis was performed to obtain the LD_{50} value of each of the samples.

III. RESULTS

Initial experiments used both purified and partially purified dried toxin, using material weight as a basis in comparing samples assayed. However, subsequent work utilized only the partially purified dried toxin because it was more readily available and more stable. Crystalline toxin prepared in concentrations ranging from 1 to 1,000 μg/ml was tested to obtain the system range. The most linear curve could be plotted from concentrations between 5 to 50 μg/ml. A typical hemagglutination titer of purified toxin is shown in Figure 2. When the partially purified dried toxin was tested in a similar manner, the agglutinating activity was approximately 10 to 11% of that of pure toxin. Consequently, the operational concentration was increased to a range between 50 to 500 μg/ml, total weight basis.

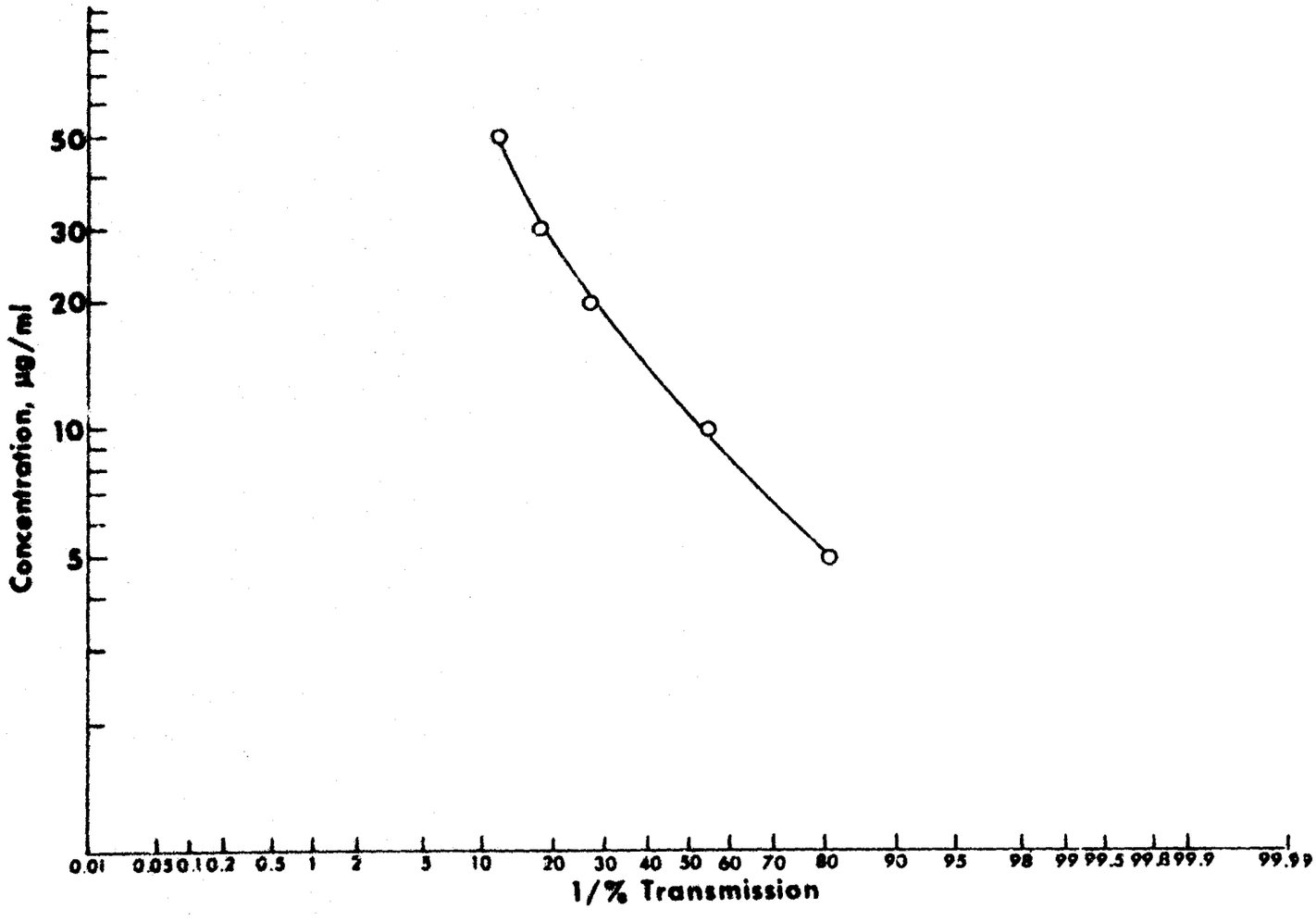

Figure 2. A Typical Hemagglutination Titer of Purified Type A Botulinum Toxin using Rabbit Erythrocytes. Procedure performed in the Autoanalyzer HA system.

Standard curves for hemagglutination values were prepared in the automated system for 50, 100, 200, 300, and 500 μg/ml concentrations of the partially purified toxin. Dilutions from four identical 0.1-g samples of this toxin were assayed in mice, and a mean value of 4.5×10^9 $MIPLD_{50}/g$ was obtained. For purified material, the toxicity reported by Schantz[2] was $2.4 \times 10^8 \pm 20\%$ $MIPLD_{50}/mg$ N. Using the conversion factor of 1,000/6.25, the toxicity per gram of crystalline toxin would be 3.8×10^{10} $MIPLD_{50}$. This compares favorably with the hemagglutination titer of the partially pure toxin; i.e., $4.5 \times 10^9 / 3.8 \times 10^{10}$ is approximately 12%. LD_{50} values were assigned to the standard curves for relating material weight with probit values. By correlating these values, it was unnecessary to compensate for the indirect proportions between constant volumes of erythrocytes and increases in hemagglutinin titer that were observed by Lowenthal[5] and Lamanna.[4] Other factors suggested by those authors as affecting the hemagglutinin-toxicity relationships were easily controlled in this system. All volumes, reaction times, and temperatures were held constant. A stable pH of 6.8 was obtained by using the PBS in preparing all reagents and dilutions of samples. To ensure that temperature outside the system had no effect, all samples were prepared and assayed immediately under identical conditions either in the hemagglutination system or in mice for confirmatory tests.

The accuracy and reproducibility of the automated system in performing hemagglutination tests were statistically analyzed. Four carefully weighed 0.1-g samples of the partially purified dried material were dissolved in a known amount of PBS and then divided into two subsamples. Each of these samples was used to prepare 50, 100, 200, 300, and 500 μg/ml concentrations of the material. These concentrations were assayed twice so that, for each point in a standard curve, there was a total of sixteen replicates. Confidence limits for the mean of each concentration tested, expressed in per cent light transmission, are shown in Table 1. The analysis of variance (Table 2) showed that there was no statistically significant variation (95% confidence) among samples or between aliquots within samples.

TABLE 1. CONFIDENCE LIMITS FOR THE MEANS OF HEMAGGLUTINATION VALUES FOR 16 REPLICATES USED IN PREPARING STANDARD CURVES

Concentration, μg/ml	Mean, Per Cent Light Trans.	95% Confidence Interval of the Mean, Per Cent Light Trans.
50	10.96	±0.25
100	20.62	±1.56
200	41.70	±3.96
300	60.12	±4.88
500	77.31	±4.74

TABLE 2. ANALYSIS OF VARIANCE IN MEASURING HEMAGGLUTINATION[7,8]

	SS		df	MS		F	
Intercepts							
Samples	0.0167	2272	3	0.0055	7424	2.38	N.S.
Aliquots	0.0093	5543	4	0.0023	3886	2.07	N.S.
Readings	0.0090	50.81	8	0.0011	3135		
Total	0.0351	2896	15				
Coefficients							
Samples	0.00179	59513	3	0.00059	86504		N.S.
Aliquots	0.00202	29167	4	0.00050	57292	1.18	N.S.
Readings	0.00665	00459	8	0.00083	12557	<1	
Total	0.01046	89139	15				

In order to show that agglutination of the erythrocytes was actually being caused by the toxin, specific antitoxin was serially diluted with PBS and mixed in equal volumes with partially purified toxin at 250 µg/ml. The results of this test, illustrated in Figure 3, showed that no agglutination occurred in the presence of specific antitoxin except for those dilutions in which complete neutralization apparently did not occur. This conclusion was substantiated by mouse tests in the following experiment. Each of these toxin-antitoxin samples were inoculated intraperitoneally into 10 groups of five mice each. Those samples that showed a slight hemagglutination reaction with 1:8 and 1:16 dilutions of specific antitoxin killed the challenged mice within 12 hours. The samples in which agglutination was definitely inhibited had no effect on the mice. The data are shown in Figure 3. This result appears to be at variance with Lamanna and Lowenthal's data,[1] which indicated that type A antitoxin neutralizes hemagglutinins and toxin disproportionately.

The possibility of using this hemagglutination system in estimating the toxic activity in liquid cultures was also investigated. Type A toxin was produced in thioglycollate and in N-Z-Amine, type B media. Samples were assayed after 3 to 4 days of bacterial cell growth in each culture. Samples of each of the cultures were prepared by removing cell debris by centrifugation for 10 minutes at 5,000 x g.

Toxin was demonstrable by hemagglutination in the N-Z-Amine culture supernatant but those samples grown in thioglycollate medium showed almost no agglutination. Evidently the hemagglutinin was partially bound with media components. When the crude toxin supernatant from the thioglycollate cultures was titrated to pH 3.5 with N HCl, a precipitate was formed. This precipitate was washed and rediluted to original volume with PBS, and agglutination occurred as expected upon assay.

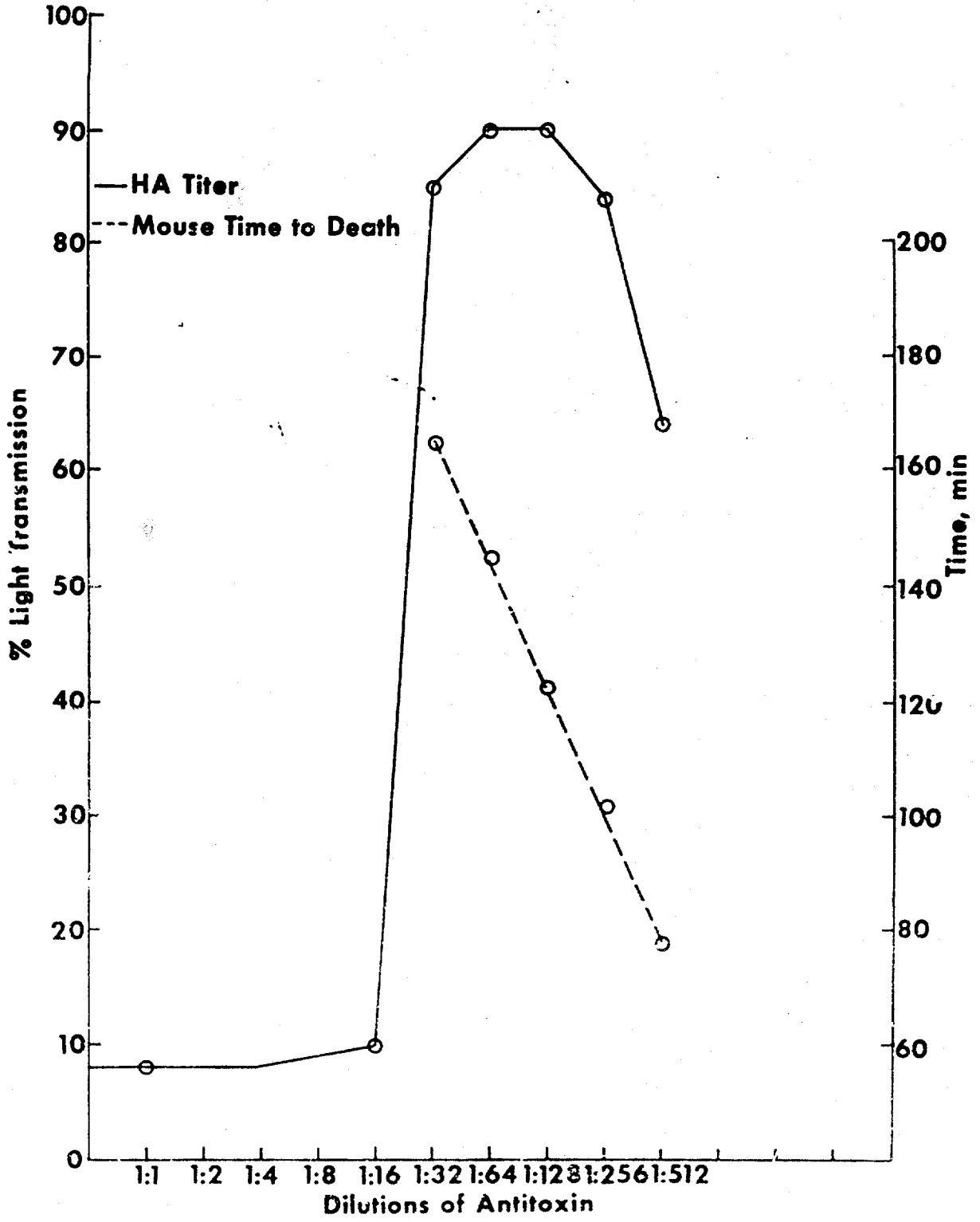

Figure 3. Inhibition of Hemagglutination of Rabbit Erythrocytes by Type A Toxin. A constant amount of toxin is plotted against increasing dilutions of specific type A antitoxin. Total time to death for IP-challenged mice is indicated. Mice challenged with 1:16 and 1:8 dilutions died within 12 hours; those inoculated at 1:4, 1:2, and 1:1 were unharmed.

To test the effect of the thioglycollate medium upon hemagglutination, a 50 μg/ml sample of purified toxin was added to an equal volume of sterile thioglycollate medium. As a control, a similar sample was prepared in PBS. Hemagglutination tests were then performed on PBS, sterile thioglycollate medium, a centrifuged thioglycollate culture supernatant, sterile thioglycollate medium plus toxin, and PBS plus toxin. These tests showed that in the presence of thioglycollate medium almost no agglutination occurred. The results are shown graphically in Figure 4. The estimated pure toxin concentrations are based upon the approximate value of 4.5×10^9 $MIPLD_{50}$/g of partially purified dried toxin.

As indicated, the N-Z-Amine, type B culture supernatant agglutinated the erythrocytes without any prior treatment of the sample. To determine if the toxin actually caused the observed agglutination, we divided the sample into two parts. One part was filtered through an EK Seitz filter before assay in either the hemagglutination system or in mice. The adsorptive qualities of these filters have been assessed.[9,10] The other part of the supernatant was tested without any treatment. The hemagglutination titer of the sample without treatment (Fig. 5) predicted a biological activity of 6.7×10^5 $MIPLD_{50}$/ml. When inoculated at a 10^5 dilution into a group of 10 mice, five died in 4 days. The sample that passed through the Seitz filter showed no agglutination (Fig. 5) and had no effect when inoculated into mice.

IV. DISCUSSION

Good agreement was obtained between hemagglutinin titer and toxicity of pure and partially purified toxin. When considered on a toxin-to-weight ratio, the hemagglutinin titer of partially purified toxin indicated that it was approximately 10 to 11% of that of pure toxin. When assayed in animals, the percentage of toxicity of the partially purified toxin was approximately 11.8% by weight.

The addition of specific antitoxin to the toxic samples tested showed that a definite relationship existed between hemagglutinin titer and toxicity. Where hemagglutination was completely inhibited by antitoxin, there was no effect on laboratory animals when inoculated with these mixtures. When the test animals were inoculated with the samples in which inhibition was incomplete, the progressive time to death data shown in Figure 3 were observed.

A set of experiments was performed to establish what relationship existed between samples of known toxicity and those of culture supernatants. The indications were that supernatant samples could be estimated provided substances such as those present in a thioglycollate medium were not present.

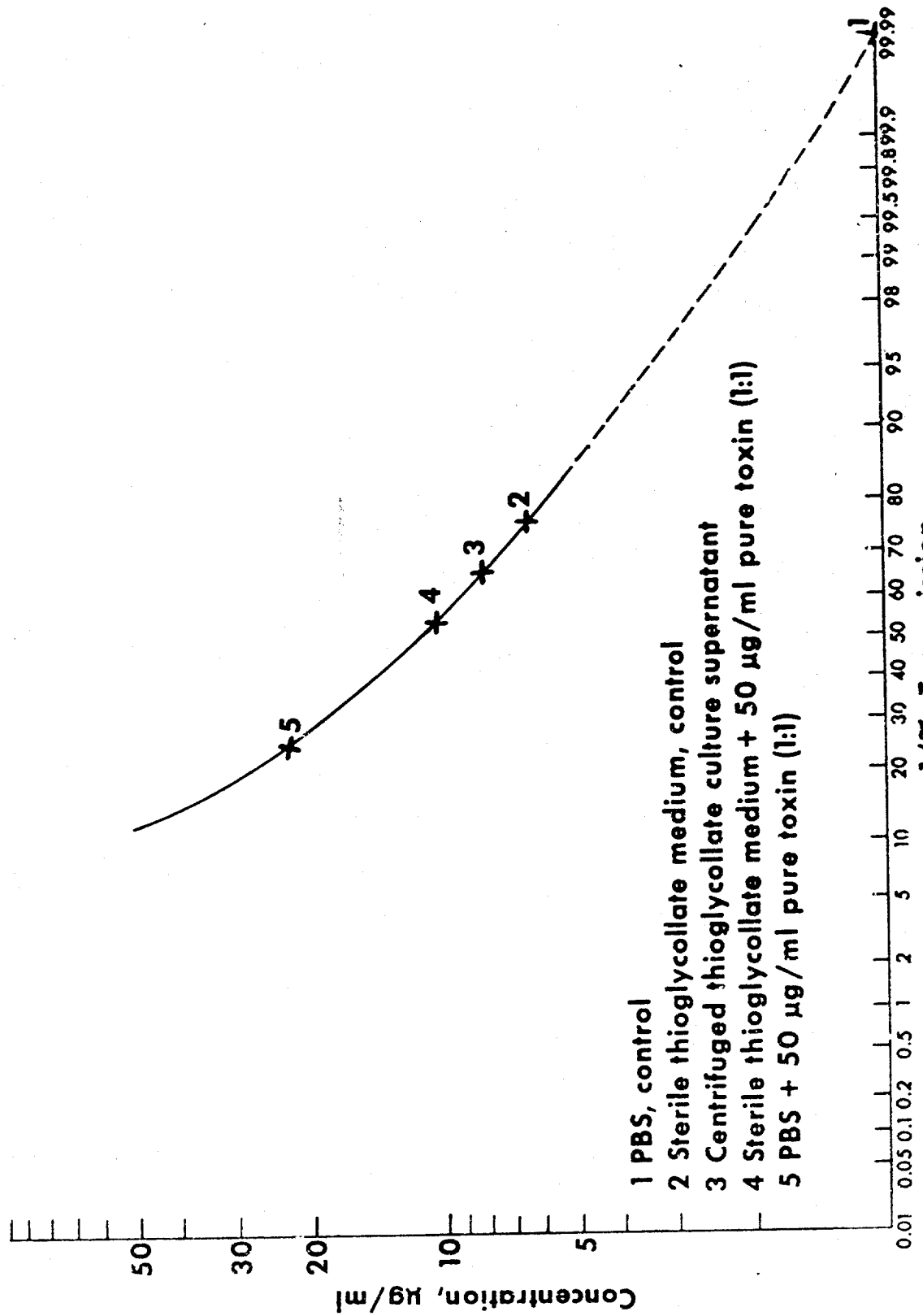

Figure 4. Effect of Thioglycollate Medium on Hemagglutination Titer of Type A Botulinum Toxin.

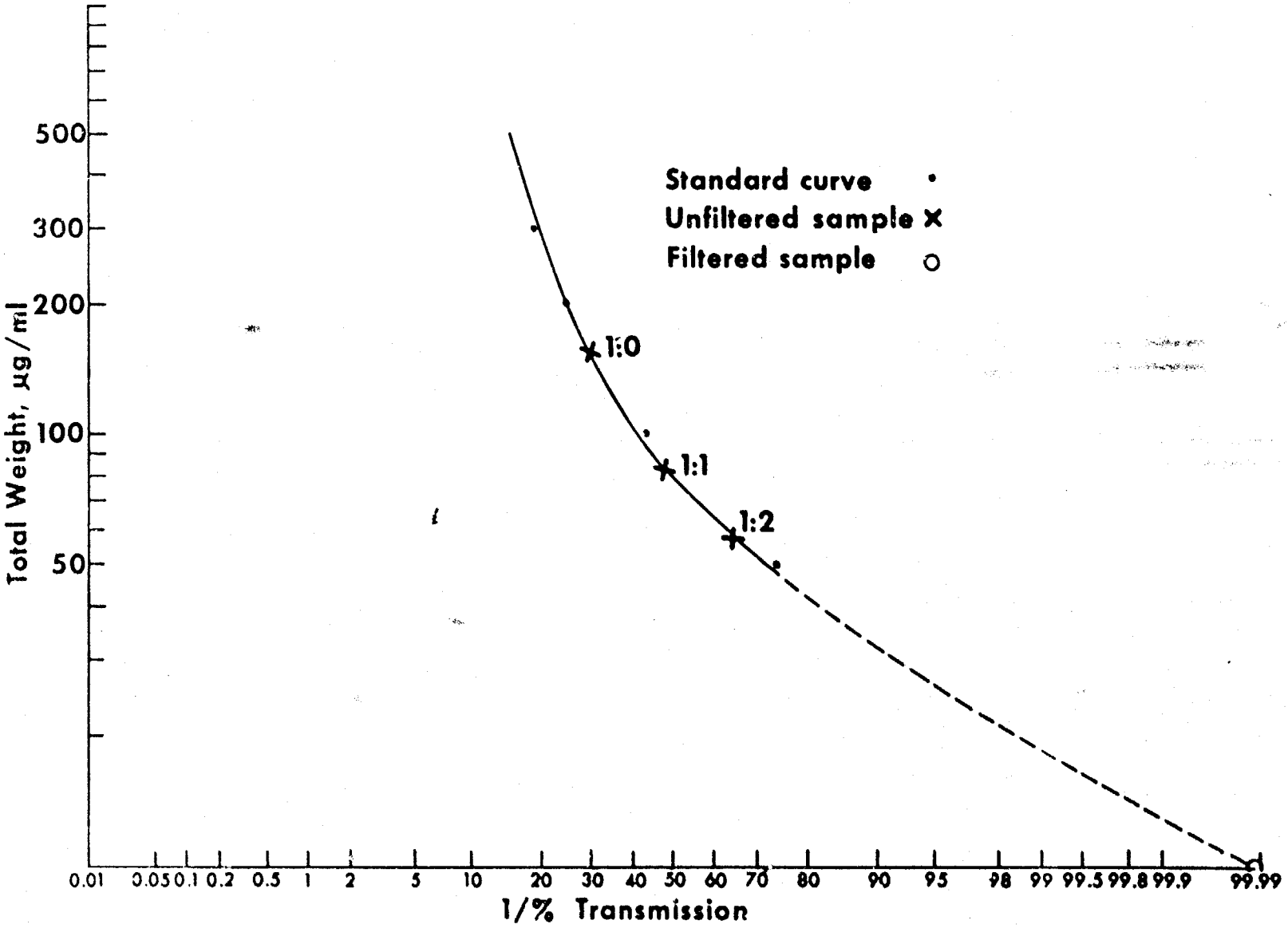

Figure 5. Comparison of Hemagglutination Values Obtained on Filtered and Unfiltered N-Z-Amine, Type B Culture Supernatants Containing Type A Botulinum Toxin. Conversion unit: 1 µg partially purified dried toxin equivalent to approximately 4.5×10^9 $MIPLD_{30}$.

This system may have application in both the detection and quantitative estimation of type A botulinum toxin. If a material is suspected of containing toxin, a hemagglutination reaction within the system would indicate qualitatively that toxin may be present. Reaction with specific antitoxin would determine whether agglutination was caused by a type A toxic product or was nonspecific. Hemagglutination cross reactions with type B toxin are recognized. In this respect, further testing is needed to determine whether agglutination inhibition is the same with type B antitoxin, or whether differences in inhibition patterns between types can be detected in the automated system. Vermilyea, Walker, and Ayres[11] observed no cross reactions between type A and type E or type B and type E, using the Ouchterlony technique. Botulinal types C and D toxicoses are nearly nonexistent in humans. Therapy after defined symptoms usually consists of administration of bivalent A-B antitoxin. It has been suggested that therapy for suspected botulinum poisoning include either the A-B-E antitoxin mixture, or the pentavalent human antitoxin.[2] In any event, the hemagglutination phenomena permit a rapid presumptive test for at least type A toxin. Demonstrable presence of toxin in quantitative values would be of considerable value therapeutically, because the complete procedure for an unknown can be performed in 20 minutes.

The hemagglutination procedure may also have use in titrating the protective strength of specific antisera. Inhibition of agglutination by serially diluted antitoxin, using a standardized toxin or toxoid, could be used to determine the relative strengths of various antitoxic sera. Conversely, if the potency of the antitoxin is known, it is possible to relate toxicity of an unknown sample in terms relative to the antitoxin.

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DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Department of the Army Fort Detrick, Frederick, Maryland, 21701	2a. REPORT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3. REPORT TITLE

AN AUTOMATED HEMAGGLUTINATION TECHNIQUE FOR ESTIMATING THE ACTIVITY OF TYPE A BOTULINUM TOXIN

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)

James B. Williams

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
June 1968	21	11

8a. CONTRACT OR GRANT NO. b. PROJECT NO. 1B522301A082 c. d.	9a. ORIGINATOR'S REPORT NUMBER(S) Technical Manuscript 469 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

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13. ABSTRACT

A quantitative relationship between hemagglutination titer and animal toxicity of type A botulinum toxin was demonstrated utilizing a modification of the hemagglutination procedure of Rosenfield et al. Hemagglutination titer was correlated to animal toxicity by measuring hemagglutinin activity of known concentrations of toxic material with an established mouse intraperitoneal LD_{5c} value. The automated procedure employed is accurate and reproducible in the range of 5 to 50 μg toxin per ml.

14. Key Words

Hemagglutination
Botulinum toxin
Toxicity
Antitoxin

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